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(71) Applicant: THOMAS JEFFERSON UNIVERSITY [US/US]; 1020 Walnut St Suite 630, Philadelphia, PA 19107 (US).

(72) Inventor: PONNAPPA, Biddanda, C.; 325 Scola Road, Brookhaven, PA 19015 (US).

(74) Agent: MONACO, Daniel, A.; Drinker, Biddle & Reath, LLP, One Logan Square, 18th and Cherry Streets, Philadelphia, PA 19103-6996 (US).

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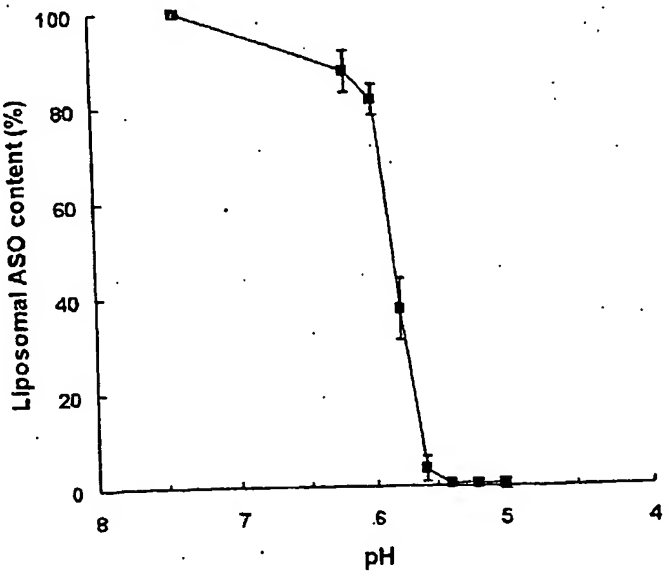
(54) Title: PH SENSITIVE LIPOSOMAL DRUG DELIVERY



(57) Abstract: The present invention discloses a novel liposome composition wherein phosphatidyl ethanolamine, cholesteryl hemisuccinate, and cholesterol in a ratio of 7:4:2 allow for the efficacious administration of a therapeutic agent to a macrophage. The liposomes of the present invention are stable at physiological pHs, while at the same time being fusogenic at acidic pHs. This property allows for the delivery of the therapeutic agent into the cytosol, and subsequently the nucleus, of the macrophage. The liposome composition disclosed herein is useful in the treatment of macrophage associated diseases or conditions.

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Kupffer cells prior to administration of hepatotoxins prevents liver damage. (Adachi, Y., et al., *Hepatology* 20: 453-460, 1994; Laskin, D.L., et al, *Hepatology* 21: 1045-1050, 1995; Ishiyama, H., et al., *Pharmacol. Toxicol.* 77: 293-298, 1995). Tumor necrosis factor alpha (TNF-α), a pro-inflammatory cytokine, exhibits pleiotropic effects on various cell types. (Beutler, B., Cerami, A., *Nature* 320:, 584-588, 1986). Kupffer cells are the major producers of TNF-α following exposure to lipopolysaccharide (LPS), the bacterial endotoxin. (Decker, K., *Eur. J. Biochem.* 192: 245-261, 1990). An over-production of TNF-α has been associated with the development of alcoholic liver injury (McClain, C.J., Cohen, D.A., *Hepatology* 9: 349-351, 1989; Nanji, A.A., et al., *Hepatology* 19: 1483-1487, 1994; Kamimura, S., Tsukamoto, H., *Hepatology* 21: 1304-1309, 1995), rheumatoid arthritis (Elliot, M.J., et al., *Lancet* 344:, 1105-1110, 1994), inflammatory bowel disease (Miurch, S.H., et al., *Gut* 34: 1705-1709, 1993), and septic shock (Michie, H.R., et al., *N. Engl. J. Med.* 318: 1481-1486, 1988). Antibodies which bind TNF-α, neutralize the effects of TNF-α released by Kupffer cells in conditions such as ischemia reperfusion (Wanner, G.A., et al., *Shock* 11: 391-395, 1999) and experimental liver damage induced by chronic alcohol consumption. (Iimuro Y., et al., *Hepatology* 26: 1530-1537, 1997).

In recent years, the use of antisense oligodeoxynucleotides (ASOs) has been an alternative approach to suppress the synthesis of specific proteins. ASOs contain sequences complementary to RNAs, which block or destroy the targeted mRNAs (Matteucci, M.D., Wagner, R.W., *Nature* 384: S20-22, 1994; Tu, G-C., et al., *J. Biol. Chem.* 273: 25125-25131, 1998). Recently, a highly effective phosphorothioate-modified ASO was developed, TJU-2755 (Tu, G-C., et al., *J. Biol. Chem.* 273: 25125-25131, 1998), against rat TNF-α. (Tu, G-C., et al., *J. Biol. Chem.* 273: 25125-25131, 1998). While TJU-2755 (**SEQ. ID. NO: 1**) was highly effective in primary Kupffer cells *ex vivo* (>90% inhibition

to develop alternative formulations of anionic liposomes, which at low pHs, will destabilize the endosomal/lysosomal barrier such that pharmacologically relevant concentrations of a therapuetic agent are released into the cytosol and nucleus. The formulation of the present invention uses phosphatidyl ethanolamine, which is fusogenic at acidic pHs (Ellens, H., et al., Biochemistry 23: 1532-1538, 1984) cholesteryl hemisuccinate which stabilizes the liposomes at physiologic pH, and cholesterol, a membrane stabilizing agent. Based on previous observations (Ellens, H., et al, *Biochemistry* 23: 1532-1538, 1984; Straubinger, R.M., *Methods in Enzymology* 221: 361-376, 1993; Connor, J., Huang, L., *J. Cell Biol.* 101: 582-589, 1985; Tschiakowsky, K., Brain, J.D., *Shock* 1: 401-407, 1994), it is expected that upon endocytosis and acidification by a proton pump in the membrane, pH-sensitive liposomes will fuse with the endosomal membrane and destabilize the endosomal compartment resulting in the release of the contents into the cytosol. The mechanism of delivery of macromolecules into the cytoplasm by pH-sensitive liposomes has been reviewed. (Straubinger, R.M., *Methods in Enzymology* 221: 361-376, 1993). Rapid entry of ASOs from the cytoplasm into the nucleus has been reported. (Fisher, T.L., et al., *Nuclei Acid Res.* 21: 3857-3854, 1993).

The present invention discloses herein the *in vivo* efficacy of the liposome formulation by using an anti-TNF-α ASO, TJU-2755 (**SEQ. ID. NO: 1**), as an example of a therapeutic agent that is delivered to the interior of a macrophage. Following encapsulation in pH-sensitive liposomes, *in vivo* delivery is accomplished by intravenous injection. Rats are subsequently administered LPS, following which plasma TNF-α levels and the ability of liver slices incubated *ex vivo* to produce TNF-α are determined. Results show that ASO TJU-2755 (**SEQ. ID. NO: 1**) effectively inhibits the ability of the liver to produce TNF-α and lowers plasma TNF-α levels, demonstrating a therapeutic potential of this delivery system *in vivo*. The use of ASO TJU-2755 (**SEQ. ID. NO: 1**) as a therapeutic agent delivered to macrophages is

cholesteryl hemisuccinate, and cholesterol in a molar ratio of 7:4:2. In another embodiment the acidic pHs comprise a pH between 5 and 6. In a further embodiment the physiological pHs are a pH between 7 and 8. In another embodiment the liposome has a diameter between 0.2-2.0 μ.

It is an object of the present invention to provide a pharmaceutical composition that includes a pharmaceutically acceptable carrier and a liposome, wherein the liposome has a lipid component including a phosphatidyl ethanolamine, a cholesteryl hemisuccinate, and cholesterol component.

It is another object of the present invention to provide a method of treating a macrophage associated disease or condition in a mammal. A therapeutically active agent is encapsulated into a liposome having lipid components which include a phosphatidyl ethanolamine, a cholesteryl hemisuccinate, and a cholesterol. The phosphatidyl ethanolamine component renders the liposome fusogenic at acidic pHs and the cholesteryl hemisuccinate component, together with the cholesterol component, renders the liposome stable at physiological pH. The liposome is administered to the mammal, thereby delivering the liposome to a macrophage. The liposome is taken up by the macrophage, and subsequently fuses with a lysosome in the macrophage, thereby destabilizing the liposome fused with the lysosome in the macrophage, and releasing the therapeutic agent into the cytosol of the macrophage.

DESCRIPTION OF THE FIGURES

Figure 1. Stability of pH-sensitive liposomes. Liposomes encapsulated with trace amounts of labeled (^{32}P) ASO TJU-2755 (**SEQ. ID. NO: 1**) plus 100 μg of unlabeled TJU-2755 (**SEQ. ID. NO: 1**) are prepared by the reverse phase

injected with pH-sensitive liposomes encapsulated with TJU-2755 (1.5-1.75 mg/Kg body wt). At different times (24, 48 and 72 h) after ASO administration, LPS (50μg/Kg body weight for 90 minutes) is administered intravenously (i.v.) and the animals are sacrificed 90 min after this challenge. Liver is excised, slices prepared and incubated for 2 h (*infra*). The amount of TNF-α released into the medium is measured by ELISA. The TNF-α values (bars) from the ASO-treated animals are normalized to control values (control=100) obtained from body weight-matched rats treated similarly except that they are injected with "empty" liposomes. Values indicated are means ± S.E. of (n) determinations. Number of animals, 24 h, n=3; 48 h, n=4, 72 h, n=3. * $p < 0.05$ (vs Control).

Figure 5. *In vivo* efficacy of ASO TJU-2755 (**SEQ. ID. NO: 1**) against TNF-α in the liver: Effect of multiple injections. Rats are injected with either one or two daily doses of ASO (1.5-1.75 mg/Kg body wt) encapsulated liposomes. Forty eight hours after the last injection, they are administered LPS (50μg/Kg) and the animals are sacrificed 90 min after this challenge. The amount of TNF-α produced by liver slices is determined as described in legend to **Figure 4** (*supra*). Bars indicate the amount of TNF-α produced by ASO-treated animals, expressed as percent of controls injected with "empty" liposomes and are the means ± S.E. of n=4 for single dose and n=7 for two doses. *$p < 0.05$, **$p < 0.01$ vs control). The absolute values (Mean) of TNF-α for control and TNF-2755-treated livers (for two daily doses) were 17,748 and 7,500 pg/g liver, respectively.

Figure 6. Effect of multiple doses of ASO TJU-2755 (SEQ. ID. NO: 1) on plasma TNF-α. Rats are injected with either one (n=3), two (n=8) or three (n=1) daily doses of TJU-2755 (SEQ. ID. NO: 1) and administered LPS (50 μg/Kg body wt.) at 48 h after the last injection of the antisense formulation as

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DESCRIPTION OF THE INVENTION

Materials and Methods

Animals: All animal experiments are carried out in male Sprague Dawley rats purchased from Harlan Sprague Dawley Inc., Indianapolis, IN. Animals are maintained on laboratory-chow. The body weights of the animals ranged from 250-300 g. The experimental protocol is approved by the Institutional Animal Care and Use Committee of Thomas Jefferson University, Philadelphia.

Chemicals: Cholesterol, cholesteryl hemisuccinate (CHEMS), dipalmitoyl phosphatidy choline (DPPC) and dipalmitoyl phosphatidy glycerol (DPPG) are purchased from Sigma Chemicals Co., St. Louis, MO. Phosphatidyl ethanolamine (PE) (transphosphatidylated from egg lecithin) is purchased from Avanti Polar Lipids Inc., (Alabaster, AL). Radioisotope [γ-^{32}P]-ATP, is purchased from Amersham Corp (Arlington Heights, IL). The phosphorothioate oligonucleotides used are custom synthesized either from Geneset Inc (La Jolla, CA), or from Hybridon Inc., (Milford, MA). Essentially two types of oligonucleotides are used in the present invention and both are 21 nucleotides long. The first one, ASO TJU-2755 (**SEQ. ID. NO: 1**), had a sequence (5'-TGATCCACTCCCCCCTCCACT-3'; **SEQ. ID. NO: 1**), complementary to the 3'- untranslated region of rat TNF-α mRNA. (Tu *et al.*, 1998). The second oligo, TJU-2755SS (**SEQ. ID. NO: 2**), was a "sense" oligonucleotide, complementary to TJU-2755 (**SEQ. ID. NO: 1**) (Tu, G-C., et al., *J. Biol. Chem.* 273: 25125-25131, 1998). All other chemicals used are of reagent grade, purchased either from Sigma Chemicals Co. or from Fisher Scientific, Pittsburgh, PA.

the liposomal preparation is treated with a mixture of chloroform and methanol (1:1 v/v) to release the contents. The solvent is evaporated and the oligonucleotide is extracted in TE buffer. The amount of ASO in solution is quantitated spectrophotometrically or by Southern hybridization (*infra*). Depending upon the concentration of ASO used, the encapsulation efficiency varies from 15-75%. Routinely, for 20-25 mg of ASO, the encapsulation efficiency ranges from 17-20%.

pH-sensitivity of liposomes: Liposomes are prepared (*supra*) encapsulating trace amounts of (^{32}P)-TJU-2755 (SEQ. ID. NO: 1) (**SEQ. ID. NO: 1)** plus 100 μg of the unlabeled ASO. Aliquots of the liposomal suspension are exposed to varying pH conditions, in the range of pH 5-7.4 at 37°C for 15 min. The suspensions are centrifuged at 100,000 x g for 45 min at 4 °C to separate the supernatant from the liposomes. The amount of radioactivity released into the supernatant is expressed as percent of the radioactivity that is present in the total suspension.

Liposomal storage stability and size determination studies: To determine the stability of the liposomes upon storage, trace amounts of TJU-2755 (**SEQ. ID. NO: 1**) are labeled with [γ-^{32}P]ATP (*supra*) and mixed with 2 mg of the unlabeled ASO. Liposomes are prepared (*supra*) and stored at 0-4° C. At various intervals up to 4 weeks, aliquots of the liposomal suspension are centrifuged at 100,000 x g for 45 min to separate the liposomes from the medium. The amount of radioactivity retained in the liposomes is compared with that present on the day of preparation, "day zero".

The mean liposome diameter is determined by Quasi-elastic light scattering using Coulter N4MD, Hialeah, FL. An Unimodal (cumulants) fit to log-Gaussian distribution is applied to determine the mean particle size (Uster, P.S., et al., *FEBS Letters* 386: 243-246, 1996). The measurements are

However, unless indicated otherwise, the amount of TJU-2755 (**SEQ. ID. NO: 1**) injected ranged between 1.5-2.0 mg/kg body weight. Rats are injected single or multiple daily doses of the ASO, and sacrificed 24, 48 or 72 h post injection, as per specific experiments detailed in figures legends. Ninety minutes prior to sacrifice, animals are administered LPS (50 μg/kg body weight). Venous blood is collected in a heparinized tube 90 min post-injection and is kept on ice until processed for TNF-α. Liver, and when necessary, spleen are taken for processing (*infra*).

TNF-α secretion in the liver: Following the *in vivo* administration of LPS, the production of TNF-α in the liver is assessed by measuring the amount of TNF-α secreted by liver slices. Liver slices are prepared and incubated in culture medium according to the procedure described by Videla and Israel (Videla, L., Israel, Y., *Biochem J.* 118: 275-281, 1970) with modifications, which include the addition of insulin and fetal calf serum to the culture medium. Briefly, liver slices of uniform thickness (10 x 4 x 0.4 mm) are prepared from the mid lobe and initially rinsed in ice-cold William's E medium. The slices (2 slices per dish) are transferred to culture dishes (35 mm) containing 2 ml of fresh medium (90% William's E, 10% fetal calf serum and 2 units of insulin/100 ml) and incubated at 37°C for 2 h. The amount of TNF-α released into the medium is measured (*infra*).

Enzyme-linked Immunoabsorbent Assay (ELISA) of TNF-α: ELISA is conducted by using Cytoscreen KRC3012 kits (Biosource, Camarillo, CA) according to the manufacturer's specifications. Supernatants containing high TNF-α levels are diluted prior to the assay to assure assay results within the standard curve.

Palo Alto, CA) and hybridized for 3 h in fresh buffer containing labeled (P-32) probe, a 21-mer oligonucleotide TJU-2755SS (**SEQ. ID. NO: 2**) complementary to TJU-2755 (**SEQ. ID. NO: 1**). Routinely, the isotope concentration in the hybridization buffer is 2-3 x 10^6 cpm/ml. Following hybridization, the membrane is washed in 5X SSC (10 min) and 5X SSC/0.05% SDS (5 min) at 40°C. The blotted membrane is placed between the folds of a Saran wrap and exposed to X-ray film overnight at -70°C. The autoradiograms are scanned (Model JX-330, Sharp Corporation, Japan, equipped with Image Scan software) and the densities associated with bands (contours) are compared. In each gel, along with the samples, varying concentrations of the standard TJU-2755 (**SEQ. ID. NO: 1**) (2.5 to 20 ng) are also included. The concentration of the unknown is extrapolated from the standard curve.

Tissue levels of TNF-α mRNA by Northern Hybridization:

Extraction of RNA: Liver and spleen samples (≈100mg) are homogenized in 3 volumes of cold TRI Reagent (Molecular Research Center, Inc.). The resultant homogenate is treated with chloroform for phase separation, precipitated with isopropanol, and washed with ethanol (70% v/v). The purified RNA pellet is resuspended in diethylpyrocarbonate (DEPC)-treated water and kept frozen until further analysis. The intactness of total RNA is measured by agarose (1%) gel electrophoresis by visualizing the intactness of ribosomal RNA (28S, 18S). Isolation of mRNA from total RNA is accomplished using Qiagen's Oligotex mRNA Spin-Column Midi Kit as per protocol. The purity of the isolated mRNA is monitored by analyzing the absorption ratios of 260 to 280 (nm), which always ranged from 1.8-2.0.

Quantitation of mRNA: Samples of mRNA are run on 1% agarose gel electrophoresis, vacuum transferred (BIORAD Model 785 Vacuum Blotter)

concentration of liposomes attained *in vivo* after intravenous injection is in a much lower range (0.5-0.6 mg lipid/ml plasma), they remain intact during the period of rapid sequestration by the macrophages, similar to that reported for other anionic liposomes. (Ponnappa, B.C., et al., *J. Liposome Res.* 8: 521-535, 1998).

*In vivo efficacy of ASO TJU-2755 (**SEQ. ID. NO: 1**)*

Delivery of liposome contents is readily assessed by ordinary skilled artisans given the teachings of this invention, using routine techniques. The efficacy of TJU-2755 (**SEQ. ID. NO: 1**), administered *in vivo*, is assessed by two different methods. One measures the amount of TNF-α produced by the liver tissue itself and the other determines plasma TNF-α levels. In both cases, the animals are administered LPS prior to determination of TNF-α. Based on preliminary observations, it is expected that there will be a time lag between the phagocytosis of liposomes by the macrophages and the entry of the oligonucleotides into the nucleus. In the initial studies, the effect of a single intravenous administration of ASO TJU-2755 (**SEQ. ID. NO: 1**) on LPS-induced production of TNF-α is determined in liver slices. The studies show that maximal efficacy (~30% inhibition) is observed when LPS is given 48 h post injection (**Figure 4**). Therefore, in subsequent studies, the effect of multiple daily injections of TJU-2755 (**SEQ. ID. NO: 1**) is tested 48 h after the last LPS injection. As shown in **Figure 5**, the extent of inhibition increases with the number of daily doses, reaching 50% after two daily doses ($p<0.01$). A more pronounced reduction (68%, $p<0.001$, $n=8$) in TNF-α levels is observed in the corresponding plasma samples following 2 doses of TJU-2755 (**SEQ. ID. NO: 1**) (**Figure 6**). The plasma TNF-α values (LPS treated) for rats administered "empty" liposomes at 15-17 mg lipid /Kg body weight are not significantly different from those for PBS injected controls.

concentration of 5.8 μg/g tissue of liver. Under similar conditions, two daily doses of the ASO led to an accumulation of 14.7 μg/g tissue and three daily doses have an accumulation of 32.3 μg/g tissue.

TNF-α mRNA levels following the administration of ASO TJU-2755 (SEQ. ID. NO: 1)

Since, it has been reported that binding of ASOs to the target mRNA transcripts initiated RNase H-mediated degradation of mRNA (Giles *et al.*, 1995), the inhibitory effects of TJU-2755 (**SEQ. ID. NO: 1**) on LPS-induced TNF-α production *in viv*, could also be due to reduced levels of TNF-α mRNA, as reported earlier in isolated Kupffer cells. (Tu, G-C, et al, *J. Biol. Chem.* 273: 54-58, 1998). As shown in **Figure 8**, in the liver tissue, there is a 35% reduction ($p < 0.01$) in the steady-state levels TNF-α mRNA in the group pre-treated with TJU-2755 (**SEQ. ID. NO: 1**) as compared to controls ("empty" liposomes). It is reasonable to assume that LPS-induced changes in TNF-α mRNA in the whole liver tissue reflects, to a large extent but not exclusively, effects on Kupffer cells. Similar to the liver, the spleen also contains of macrophages which are targeted by anionic liposomes. (Ponnappa, B.C., et al., *J. Liposome Res.* 8: 521-535, 1998). For comparative purposes, TNF-α mRNA is determined in the spleen following LPS administration, and is found to be similarly affected by TJU-2755 (**SEQ. ID. NO: 1**), as shown by a 36.5% reduction ($p< 0.05$) in mRNA levels (**Figure 8**).

Therapeutic compositions

The method of the present invention can be used in one of many different mammalian species, including but not limited to, bovine, ovine, porcine, equine, rodent and human. Further provided herein is a method of delivering a therapeutic agent to a cell. "Therapeutic agents" which may be delivered by the liposomes into cells are any compound or composition of

deliver therapeutically or diagnostically effective amounts of therapeutic or diagnostic agents into the cells of a mammal afflicted with a macrophage associated disease, disorder or condition amenable to diagnosis or treatment with the agent. Hence, such delivery fusion is used to diagnose or treat the mammal for the disease, disorder or condition. For example, the mammal can be afflicted with a septic condition, an infectious microbial disease, e.g., a viral or bacterial infection, or inflammatory condition, e.g., an arthritic condition or autoimmune disorder such as rheumatoid arthritis or juvenile diabetes, and a therapeutically effective amount of an antimicrobial or anti-inflammatory agent is delivered to the mammal's cells.

The delivery of liposomal contents to cells is facilitated by the incorporation of phosphatidyl ethanolamine (PE) into the liposomes, as the PEs destabilize the liposomes' bilayers in the presence of an acidic pH, as occurs intracellularly in the lysosomes. PE-mediated destabilization of endosomal/lysosomal membranes results in direct deliverly of liposomal contents into the cells.

Pharmaceutical compositions

Also provided herein are compositions containing the liposomes of this invention. Included in such compositions are pharmaceutical compositions that also comprise a "pharmaceutically acceptable carrier," which is a medium generally acceptable for use in connection with the administration of liposomes to mammals, including humans. Pharmaceutically acceptable carriers are formulated according to a number of factors well within the knowledge of the ordinarily skilled artisan to determine and account for, including without limitation: the particular therapeutic agent used, its concentration, stability and intended bioavailability; the disease, disorder or condition being treated with the composition; the subject, its age, size and general condition; and the composition's intended route of administration.

complete instability in undiluted plasma or sera (typically, in *in vitro* studies, serum is diluted 5-20%), their usefulness for *in vivo* delivery is limited.

pH-stable anionic liposomes have been used as an efficient delivery vehicle for targeting oligonulceotides to Kupffer cells *in vivo* (Ponnappa, B.C., et al., *J. Liposome Res.* 8: 521-535, 1998); however, the liposomes used in that study do not contain the pH-sensitive fusogenic lipid, phosphatidyl ethanolamine, and therefore, do not possess the ability to destabilize the endosomal/lysosomal membrane barrier. As shown in **Table I**, ASO TJU-2755 (**SEQ. ID. NO: 1**) is ineffective when delivered in that fashion. The liposomal composition of the present invention overcomes this barrier by incorporating the pH-sensitive fusogenic lipid phosphatidyl ethanolamine, as well as cholesterol hemisuccinate to maintain stability at physiological pH, into the liposomal lipid composition, thereby fulfilling a long sought, yet unfulfilled, need to effectively deliver compounds to the interior of a cell.

Macrophages contain scavenger receptors that recognize an array of negative charges such as in anionic liposomes, thereby allowing for the efficient uptake of the liposomes of the present invention. (Ponnappa, B.C., et al., *J. Liposome Res.* 8: 521-535, 1998; Bautista, A.P., et al., *J. Leukocyte Biol.* 55: 321-327, 1994). Sequestration by macrophages, of which the Kupffer cells in the liver are an example, is further facilitated by the large size of the liposomes. (Alino, S.F., et al., *Biochem Res. Com.* 192: 174-181, 1993). The method employed in the present invention results in the formation of liposomes in which about 90% of the liposomes have a diameter larger than 200 nm, thus preventing them from crossing the fenestrations in the endothelial barrier of the liver and spleen sinusoids. This barrier has the highest permeability of tissue capillaries. As macrophages migrate towards areas of infection/inflammation, the large size of the liposomes of the present invention allows for efficient delivery of the therapeutic agent to the macrophages. The pH-sensitive liposomes of the present invention are

inhibition of TNF-α production at 48 h post injection. The slow onset of inhibition is due to a slow release of the liposomal contents from the endo-lysosomal compartment.

The post-injection duration of the antisense oligonucleotides (**Figure 4**) reveals that following a single injection, the effect of the ASO begins to fade after 72 h, suggesting gradual degradation of the oligonucleotide. Although the exact half-life of TJU-2755 (**SEQ. ID. NO: 1**) is not known, others have reported half-lives in the range of 48 h for similar phosphorothioate antisense oligonucleotides. (Saijo, Y., et al., *Oncology Res.* 6: 243-249, 1994). Consequently, multiple doses of the antisense are more effective. Indeed, successive injections of the ASO result in a cumulative increase in the tissue levels of the oligonucleotide, which is also associated with a significant inhibition of LPS-induced TNF-α production, both in the liver and plasma (**Figures 5-7**). Interestingly, with two daily doses of ASO TJU-2755 (**SEQ. ID. NO: 1**), the effect is more pronounced in the plasma (68% inhibition) than in the liver (50% inhibition). Increasing the dose and/or the number of daily administrations of the liposome encapsulated ASO will allow for greater effects, both on liver and plasma levels, as a plateau is not observed. Preliminary reports reveal that similar to the liver slices (Kupffer cells), spleen slices (splenic macrophages) have a high capacity to produce TNF-α in response to LPS stimulation, and therefore, can also raise plasma levels of TNF-α. Since spleen is simultaneously targeted during the *in vivo* delivery of anionic liposomes (Ponnappa, B.C., et al., *J. Liposome Res.* 8: 521-535, 1998), spleen-associated production of TNF-α will also be inhibited by the delivery of ASOs. Release of mediators from the spleen into the portal circulation appear to be relevant to the development of hepatocellular injury mediated by LPS. It has been reported that splenectomy significantly reduces the liver damage induced by the administration of large doses of LPS. (Hiraoka, E., et al., *Liver* 15: 35-38, 1995).

In summary, although several strategies/drugs have been developed to suppress the chronic *in vivo* production of TNF-α to control inflammatory diseases such as alcohol liver disease, rheumatoid arthritis, Crohn's disease and septic shock, the discovery of an ideal drug still remains a challenge. Antibodies targeted against TNF-α have been successfully used in some of the clinical trials, but the potential for antigenicity and toxicity remains. (Eigler, A., et al., *Immunology Today* 18: 487-492, 1997). Therefore, the efficacy of the liposomal delivery system of the present invention is of pharmacological significance.

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a) encapsulating a therapeutically active agent into a liposome having lipid components which comprise a phosphatidyl ethanolamine, a cholesteryl hemisuccinate, and a cholesterol wherein said phosphatidyl ethanolamine component renders said liposome fusogenic at acidic pHs and said cholesteryl hemisuccinate component, together with said cholesterol component, renders said liposome stable at physiological pH;

b) administering said liposome to said mammal;

c) delivering said liposome to a macrophage wherein said liposome is taken up by said macrophage;

d) fusing of said liposome with a lysosome in said macrophage;

e) destabilizing said liposome fused with said lysosome in said macrophage; and

f) releasing of said therapeutic agent into a cytosol of said macrophage.

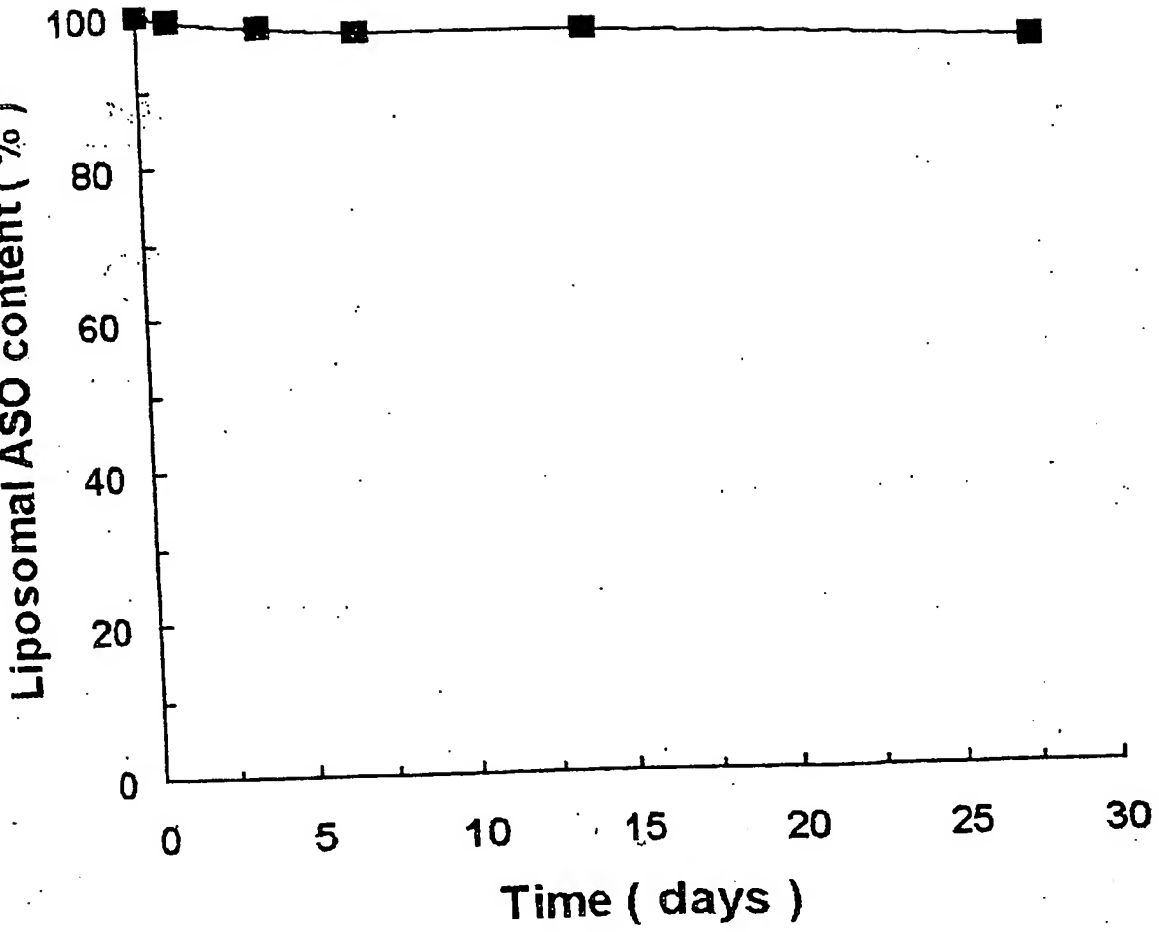
100
80
60
40
20
0
Liposomal ASO content (%)
0
5
10
15
20
25
30
Time (days)

Fig. 2

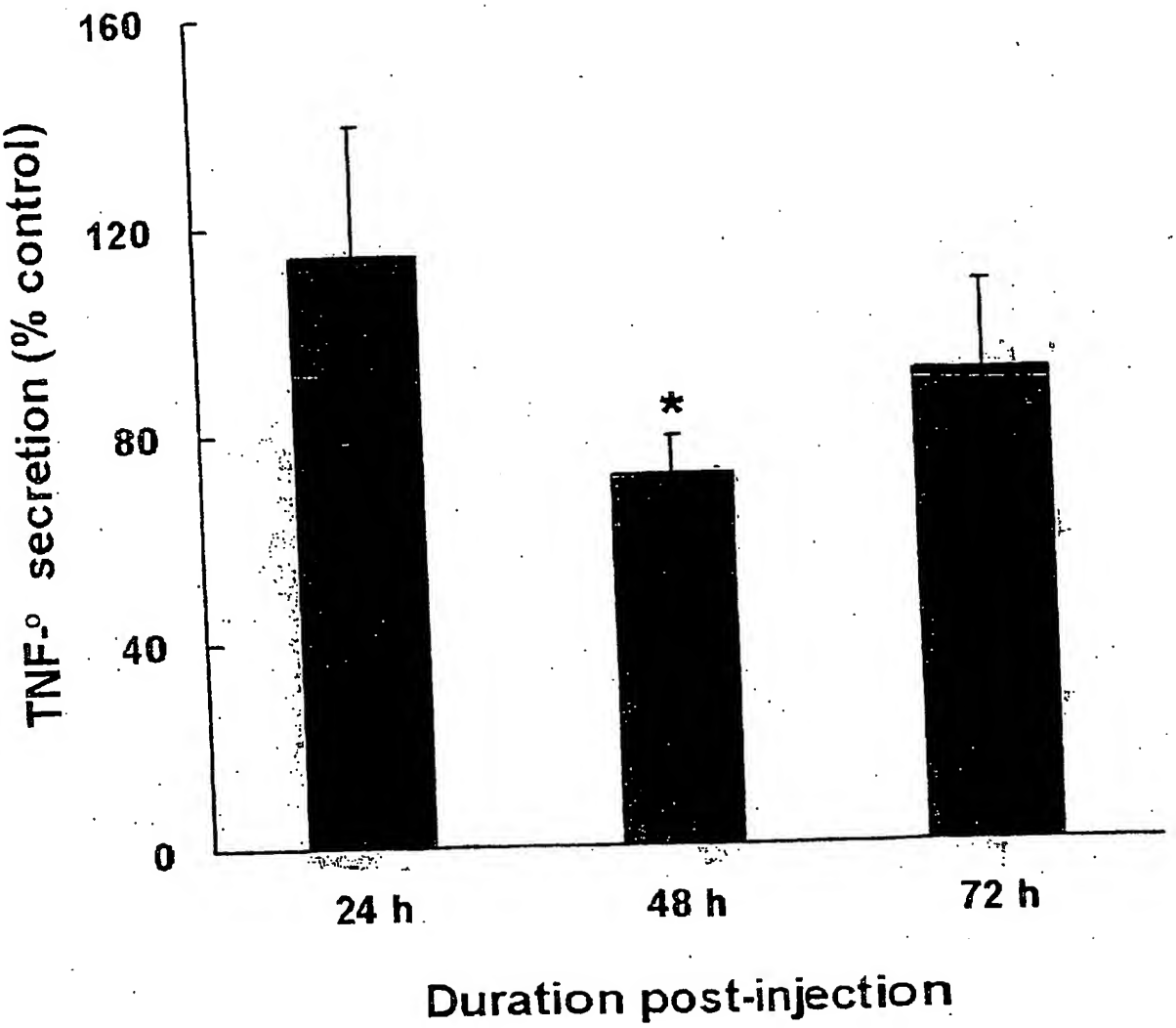
160
120
80
40
0
TNF-° secretion (% control)
*
24 h
48 h
72 h
Duration post-injection

Fig. 4

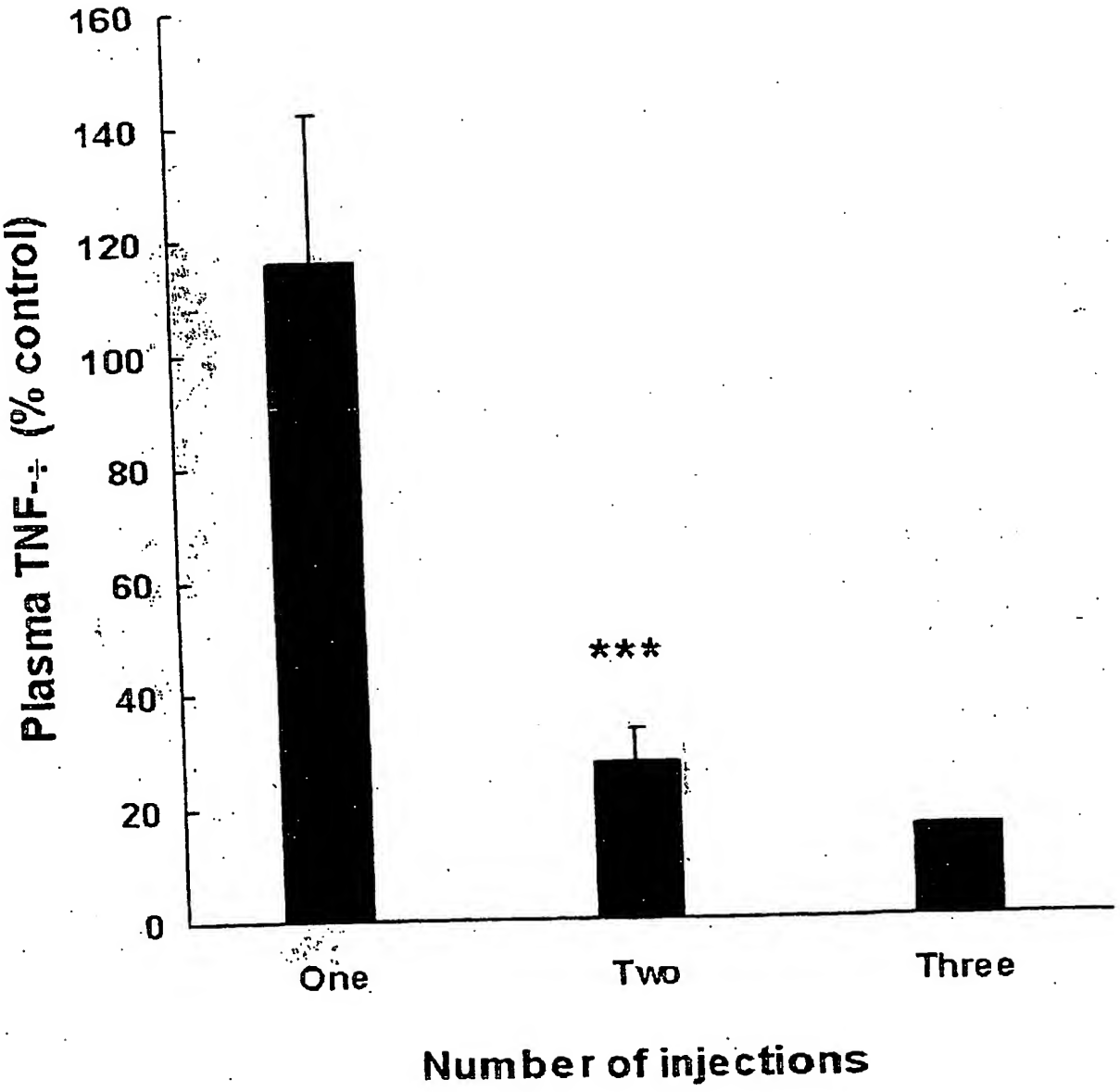
160
140
120
100
80
60
40
20
0
Plasma TNF-÷ (% control)
***
One
Two
Three
Number of injections

Fig. 6

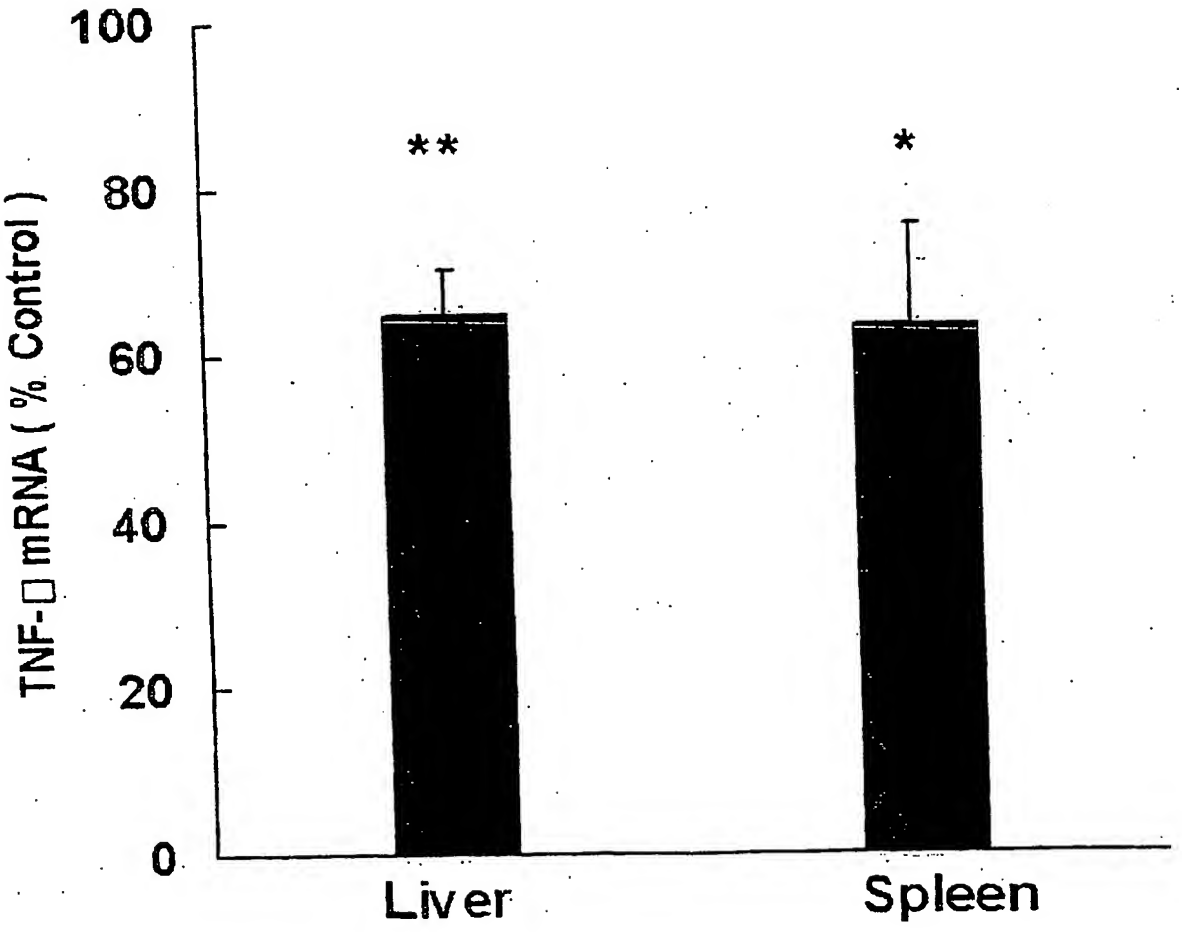

Fig. 8

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(71) Applicant: THOMAS JEFFERSON UNIVERSITY [US/US]; 1020 Walnut St Suite 630, Philadelphia, PA 19107 (US).

(72) Inventor: PONNAPPA, Biddanda, C.; 325 Scola Road, Brookhaven, PA 19015 (US).

(74) Agent: MONACO, Daniel, A.; Drinker, Biddle & Reath, LLP, One Logan Square, 18th and Cherry Streets, Philadelphia, PA 19103-6996 (US).

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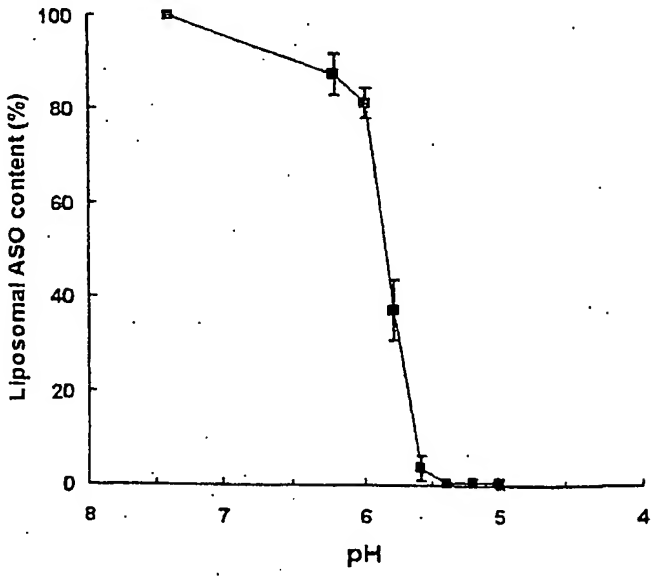
(54) Title: PH SENSITIVE LIPOSOMAL DRUG DELIVERY



(57) Abstract: The present invention discloses a novel liposome composition wherein phosphatidyl ethanolamine, cholesteryl hemisuccinate, and cholesterol in a ratio of 7:4:2 allow for the efficacious administration of a therapeutic agent to a macrophage. The liposomes of the present invention are stable at physiological pHs, while at the same time being fusogenic at acidic pHs. This property allows for the delivery of the therapeutic agent into the cytosol, and subsequently the nucleus, of the macrophage. The liposome composition disclosed herein is useful in the treatment of macrophage associated diseases or conditions.

WO 02/076427 A3

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	LEBEDEVA I ET AL: "Cellular delivery of antisense oligonucleotides" EUROPEAN JOURNAL OF PHARMACEUTICS AND BIOPHARMACEUTICS, ELSEVIER SCIENCE PUBLISHERS B.V., AMSTERDAM, NL, vol. 50, no. 1, 3 July 2000 (2000-07-03), pages 101-119, XP004257182 ISSN: 0939-6411 page 105, right-hand column, last paragraph -page 106, left-hand column, paragraph 1 ---	1-7
Y	LAI M-Z ET AL: "ACID-INDUCED AND CALCIUM-INDUCED STRUCTURAL CHANGES IN PHOSPHATIDYLETHANOLAMINE MEMBRANES STABILIZED BY CHOLESTERYL HEMISUCCINATE" BIOCHEMISTRY, vol. 24, no. 7, 1985, pages 1654-1661, XP001109500 ISSN: 0006-2960 page 1654, right-hand column, last paragraph -page 1655, left-hand column, paragraph 1 page 1656, left-hand column, paragraph 2 -page 1657, left-hand column, last paragraph page 1658, left-hand column, last paragraph -page 1660, right-hand column, paragraph 2 ---	1-7
Y	NORIO HAZEMOTO ET AL: "EFFECT OF PHOSPHATIDYLCHOLINE AND CHOLESTEROL ON PH-SENSITIVE LIPOSOMES" CHEMICAL AND PHARMACEUTICAL BULLETIN, PHARMACEUTICAL SOCIETY OF JAPAN. TOKYO, JP, vol. 41, no. 6, 1 June 1993 (1993-06-01), pages 1003-1006, XP000382889 ISSN: 0009-2363 page 1003, left-hand column, paragraph 1 -right-hand column, paragraph 1 page 1004, right-hand column, last line -page 1005, left-hand column, line 1 --- -/--	1-7

INTERNATIONAL SEARCH REPORT

Int[illegible]nal application No.
PCT/US 02/09278

Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This International Search Report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. [X] Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:

Although claim 7 is directed to a method of treatment of the human/animal body, the search has been carried out and based on the alleged effects of the composition.

2. [ ] Claims Nos.:
because they relate to parts of the International Application that do not comply with the prescribed requirements to such an extent that no meaningful International Search can be carried out, specifically:

3. [ ] Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. [ ] As all required additional search fees were timely paid by the applicant, this International Search Report covers all searchable claims.

2. [ ] As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.

3. [ ] As only some of the required additional search fees were timely paid by the applicant, this International Search Report covers only those claims for which fees were paid, specifically claims Nos.:

4. [ ] No required additional search fees were timely paid by the applicant. Consequently, this International Search Report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

[ ] The additional search fees were accompanied by the applicant's protest.

[ ] No protest accompanied the payment of additional search fees.